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Integrins and extracellular matrix (ECM) play key roles in cell migration, but the underlying mechanisms are poorly defined. Integrin α6β1, a laminin receptor, affected cell motility and induced migration onto ECM substrates with which it was not engaged. When α6A integrin was expressed in embryonic stem (ES) cells (ES6A), they extended numerous filopodia and lamellipodia, and were intensely migratory in haptotactic assays on laminin-1. Transfected α6A caused these effects, since cells transfected with control vector or α6B, a cytoplasmic domain α6 isoform, displayed compact morphology and no migration, like wild-type ES cells. The ES6A migratory phenotype persisted on Fibronectin and laminin-5. α6β1 did not contribute detectably to adhesion to these substrates in ES cells. However, anti-α6 antibodies completely blocked migration of ES6A cells on Fibronectin or Laminin-5. Cross-linking with secondary antibody overcame the inhibitory effect of anti-α6 antibodies, restoring migration or filopodia extension on Fibronectin and Laminin-5. The tetraspan cell surface molecule CD81 was shown to be physically associated with α6β1 by others. Anti-CD81 antibodies inhibited α6Aβ1-induced migration, but had no effect on ES cell adhesion. Our results suggest a mechanism by which interactions between α6Aβ1 and CD81 may upregulate cell motility, affecting migration mediated by other integrins.

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Susan Z. Romano 12/4/97

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Introduction

Cell migration is crucial to embryonic development, tissue remodeling and cancer invasion. To migrate properly, cells must integrate multiple incoming signals. Once committed to migration, they coordinately regulate, both spatially and temporally, surface receptors and cytoskeleton, in order to generate traction and movement (Lauffenburger and Horwitz, 1996). Migration usually occurs over extracellular matrix (ECM), and is accompanied by characteristic morphological changes. Cell protrusions, e.g., filopodia or lamellipodia, are sites where adherence contacts for traction generally are formed. To accomplish forward movement, there must be a balance between the establishment of plasma membrane-ECM adherence contacts at the cell leading edge, and their coordinated, asymmetric release at the cell trailing edge (Huttenlocher et al., 1995). While significant advances have been made in identifying molecules involved in cell migration, molecular mechanisms are poorly defined.

Integrins are a class of cell surface receptors that recognize ECM proteins and cellular ligands. They play an important role in migration because they can generate traction by forming mechanical transmembrane links between ECM and cytoskeleton (Lauffenburger and Horwitz, 1996). In addition, ligation of integrin receptors by ECM may transduce signals (Hynes, 1992) interfacing with a cascade of second messengers (Melchiori et al., 1995). These two aspects of integrin function are likely linked, i.e., signaling events may regulate or influence interactions with cytoskeleton or ECM, and vice versa. Integrins consist of two transmembrane subunits, α and β. To date there have been more than 14 α and 8 β subunits identified in man (Hynes, 1992). To define post-receptor occupancy events that mediate mechanical strength and signaling, considerable efforts have focused on the short cytoplasmic tails of both α and β chains. The β chain cytoplasmic tails interact directly with cytoskeletal components, e.g., talin (Horwitz et al., 1986; Knezevic et al., 1996). They may also be involved in regulating signaling (Guan et al., 1991; Lewis and Schwartz, 1995) and cell migration (Filardo et al., 1995).

The α chain cytoplasmic tails have been implicated in setting the 'activated' vs. 'resting' state of integrins, presumably via as yet undefined signaling events that affect integrin conformation (O'Toole et al., 1994). The α2 cytoplasmic tail may interact with actin directly (Kieffer et al., 1995), and the α4 cytoplasmic tail was implicated in supporting random migration, spreading and adhesion (Chan et al., 1992; Kassner et al., 1995).

The α subunits of the laminin-binding integrins α3, α6 and α7 (Hynes, 1992) are found as two isoforms, A and B, which differ by the cytoplasmic domains (Tamura et al., 1991; Mercurio, 1995). Expression of the α6A or B tail (Fig. 1), probably based on alternative exon splicing, is developmentally regulated in a position specific manner (Collo et al., 1995; Thorsteinsdottir et al., 1995). The α6Aβ1 isoform was described to induce a more migratory phenotype than α6Bβ1 in transformed macrophages (Shaw and Mercurio, 1994). The two isoforms of α7 were shown to support migration in human 293 kidney cells (Echtermeyer et al., 1996) or MCF7, human mammary epithelial cells (Yao et al., 1996), but no major differences between the isoforms were observed.

We previously showed that α6β1 is the receptor for laminin-1 (Ln-1) in mouse embryonic stem (ES) cells. We also found that undifferentiated ES cell lines, including ES1 (Cooper et al., 1991), expressed exclusively the α6B isoform. ES cells injected into blastocysts can participate in formation of any tissue (Robertson, 1987). Furthermore, they can be induced to differentiate in culture and differentiation occurs concomitantly with expression of the α6A isoform (Cooper et al., 1991). In the developing mouse embryo, α6B was the only isoform expressed until day 8.5 of development, at which time α6A became detectable, but only in the developing heart, as a gradient increasing from the outer to the inner myocardial layers (Collo et al., 1995).

In this paper, we describe that expression of α6A in ES1 cells induced filopodia extension and migration on Ln-1. Intriguingly, these effects were independent of α6A adhesive functions,

since they occurred also on substrates that did not require α6A for ES cell adhesion, i.e., Ln-5 and Fn. However, while α6A did not have to engage with an ECM ligand to induce migration, it participated in molecular interactions inhibitable by anti-α6 antibodies and mimicked by clustering. Furthermore, α6Aβ1-induced motility, but not cell adhesion, was inhibited by antibodies to CD81, a member of the tetraspan family of cell surface molecules recently found to form complexes with certain integrins including α6β1 (Berditchevski et al., 1996).

Body

Materials and Methods

Cells and transfections

ES1 cells are a subline isolated by growing D3 ES cells without a fibroblast feeder layer in the presence of leukemic inhibitory factor (LIF) on tissue culture plates coated with denatured gelatin (Cooper et al., 1991). ES1 cells were routinely passaged using 1X trypsin/EDTA (Sigma, St. Louis, MO) and grown in DMEM (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum (Gemini Bio-Products, Inc., Calabasas, CA), 0.0007% 2-mercaptoethanol, 2 mM glutamine (Irvine Scientific, Santa Ana, CA) and LIF supplied as a 1:10,000 dilution of supernatant from CHO cells transfected with LIF (Abe et al., 1991) (10% CM). Cells used for adhesion assays were harvested using 2.5 mM EDTA in HBSS (Gibco/BRL, Gaithersburg, MD) followed by extensive washing in DMEM. For expression, full length human α6A or α6B cDNA was subcloned into the expression vector, pBJneo. α6 expression was driven by the SRα promoter (Takebe et al., 1988), which is composed of the SV40 early promoter and the R-U5 region from the long terminal repeat of human T-cell leukemia virus type 1. CD8/6A and CD8/6B chimeras were constructed as described(Gaietta et al., 1994). For transfection, ES1 cells, (1×10^7), of early passage number (15-19), were trypsinized, washed twice with EP buffer (20 mM HEPES pH 7.0, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM D+ glucose, 0.1 mM 2-Me, 2.5 mM NaOH) and electroporated with 20 μg of DNA using 250 μF and 250 volts (BioRad, Hercules, CA). Cells were incubated on ice for 10 min, then cultured at 37°C, 5% CO_2 in 10% CM. After 24 h, selection media containing 300 μg/ml G418 (Gibco/BRL) was added to the cultures and changed every other day. Approximately 10-14 days post-transfection, G418-resistant colonies were trypsinized and expanded.

Flow cytometry

For FACS analysis, cells were harvested and washed with 5% fetal calf serum in HBSS. Primary mAb, BQ16, anti human α6, (Liebert et al., 1993) (ascites diluted 1:500), 2B7 (Shaw et al.,

1993) (10 μg/ml) or EA1 (Ruiz et al., 1993) (10 μg/ml), was bound to cells for 30 min on ice. Cells were washed, resuspended in goat anti-mouse-FITC (Sigma) or goat anti-rat-FITC (1:40) (Calbiochem, San Diego, CA) and incubated 30 min on ice. To detect CD8 chimera expression, phycoerythrin conjugated to anti-CD8 (Sigma) was bound to transfectants. Cells were again washed, resuspended in 2% fetal calf serum in HBSS and either analyzed on a FACScan or BQ16 or anti-CD8-stained cells were sorted on a FACSort machine (Becton Dickinson).

Morphological Analysis

Glass coverslips were coated with 20 μg/ml Ln-1 or Fn in HBSS for 30 min at 37°C. Cells (4 x 10^4) were grown in migration medium (DMEM/2 mM glutamine/1 mM sodium pyruvate/LIF) on coated coverslips overnight at 37°C. Where mAb treatments were used, cells were incubated at room temperature with 10 μg/ml GoH3 in migration medium for 30 min prior to plating. For cross-linking experiments, cells were incubated at room temperature with 10 μg/ml GoH3 for 15 min, then 10 μg/ml affinity purified goat anti-rat (Calbiochem) was added for an additional 15 min prior to plating. After 18 h, cells were washed with PBS, fixed in 2.5 % paraformaldehyde/PBS, and mounted in Immunofluore (ICN, Costa Mesa, CA). Cells were visualized in phase contrast using a Zeiss Axiovert microscope and photographed using TMAX 400 ASA film.

Monoclonal antibody production

Rats were injected with rat basophil leukemia cells (RBL) expressing human α6Aβ4 (1NA4) in PBS. Three days before spleen harvest, 1NA4 cells were injected in RIBI adjuvant (Hamilton, MT). Cells were fused to YB2/0 myeloma cells and antibody-producing cells were selected. Positive clones were identified by a cell-based ELISA assay. Briefly, 1NA4 cells were grown in 96 well plates overnight to confluency. Plates were washed with HBSS containing 5% FCS and 0.02% NaN_3. Plates were blocked with blotto (5% nonfat dried milk in PBS/ 0.2% Tween 20) and hybridoma supernatant was allowed to bind cells for 2 hr. Bound antibody was detected

using goat anti-rat-horseradish peroxidase (Amersham) secondary antibody followed by incubation with developing solution (10 mg o-phenyldiamine HCl, 10 ml 30% H_2O_2 in 25 ml of 50 mM Na_2HPO_4, 27 mM citrate, pH 5.0). Positive samples were detected reading optical density at 490 nm. A secondary screen was performed using RBL cells in the ELISA assay to eliminate mab's raised to "background" rat proteins. Positive candidates were cloned and purified on Protein G sepharose (Pierce, Rockford, IL).

Immunoprecipitation/Western Analysis

Detergent lysates were prepared from transfected ES cells, and biotinylated , RBL or 1NA4 . Briefly, cells were trypsinized, blocked with trypsin inhibitor, washed with PBS, and lysed with 2% Renex in PBS with 0.174 μg/ml PMSF, 0.7 μg/ml pepstatin A, 0.5 μg/ml leupeptin and 2 μg/ml aprotinin. Lysates were incubated on ice for 1 h then centrifuged at 40,000 rpm for 1 h. Lysates were precleared with Sepharose beads, then immunoprecipitated overnight at 4°C with the anti-β1 monoclonal antibody 9EG7 (Lenter et al., 1993), GoH3, mAb 12.2, mAb 24.9 or mAb 22.2. For immunoprecipitation, 9EG7 was covalently coupled to CNBr-activated sepharose (Pierce, Rockford, IL) and protein G sepharose was used for the remaining antibodies. Immunoprecipitated proteins were washed with 50 mM Tris HCl, pH 7.4, 0.5 mM NaCl, 0.1% Tween 20, eluted, and separated on 4-20% SDS-PAGE gradient gels (Novex, San Diego, CA) under reducing and non-reducing conditions and then transferred to PVDF (Millipore, Bedford, MA). Co-precipitation of the transfected human α6A or α6B with mouse β1 was detected using polyclonal antibodies raised against the cytoplasmic tail of α6A (6845) or α6B (0530) which had been affinity purified on an α6A peptide column (Collo et al., 1995) or similarly on an α6B peptide column. Affinity purified antibodies were detected using a secondary antibody coupled to horseradish peroxidase (Amersham, Arlington Hts., IL) followed by ECL detection. Biotinylated, immunoprecipitated proteins were detected by incuabation of avidin-horseradish peroxidase (Boehringer Mannheim) followed by ECL detection.

Migration assays

Transwell filters (8.0 μm pore size, Costar, Cambridge MA) were coated for 4 h at 37°C with varying concentrations of Ln-1 (Gibco/BRL), 40 μg/ml human Fn (Gibco/BRL), 1 μg/ml Ln-5, kind gift of Dr. M. Fitchmun, Desmos, Inc., San Diego, CA diluted in 1 mg/ml ovalbumin/HBSS. Cells (1 x 10^4 cells/filter) were plated on the uncoated side of the filter in migration medium (DMEM/2 mM glutamine/1 mM sodium pyruvate/LIF). For anti-integrin, anti-Ln-1, or anti-Ln-5 antibody blocking experiments, cells were incubated at room temperature with 10 μg/ml GoH3, 1:100 dilution of supplied concentration of affinity-purified anti-Ln-1 (Sigma), 25 μg/ml CM6 (Plopper et al., 1996), 75 μg/ml 2F7 (Boismenu et al., 1996)(hamster anti mouse CD81) kindly provided by Dr. Wendy Havran, The Scripps Research Institute, San Diego, CA or 75 μg/ml hamster anti-TNP (Phamingen, San Diego, CA) in migration medium for 30 min prior to plating on filters. For monensin treatment, ES6A, ES6B or ESneo cells in migration media, or migration media including 0.01, 0.1 or 1 μM monensin (Sigma). For cross-linking experiments, cells were incubated at room temperature with 10 μg/ml GoH3 for 15 min, then 10 μg/ml affinity purified goat anti-rat (Calbiochem) was added for an additional 15 min prior to plating on filters. Cells were maintained at 37°C in a humidified incubator containing 5% CO_2 for 18 h, then cells were fixed and stained using the Diff-Quik stain kit (Baxter, McGaw Park, IL). The uncoated side of each filter was wiped with a cotton-tipped applicator to remove cells that had not migrated though the filter. Filters were viewed under bright field optics. To quantify migration, stained cells were counted in four fields (using a 40x objective) from each of two filters for each condition. Results of representative experiments are expressed as the mean number of cells counted in each field +/- the standard deviation.

Adhesion Assays

Untreated 96 well plates (Sarstedt, Newton, NC) were coated for 4 h at room temperature with mouse Ln-1 (Gibco) (20 μg/ml), Ln-5 (1μg/ml) or with human Fn, (Gibco), (20 μg/ml). All proteins were diluted in 100 mM carbonate buffer, pH 9.3. Plates were then washed twice with phosphate buffered saline (PBS) containing 0.2% Tween 20 and blocked 1 h with blotto. Cells

were collected by treatment with 2.5 mM EDTA in HBSS, washed twice with DMEM/1% bovine serum albumin, then plated (1.2×10^5/ well) in DMEM/1% bovine serum albumin/25 mM HEPES, pH 7.2. For anti- antibody blocking experiments, cells were incubated at room temperature with 10 μg/ml GoH3 or 75 μg/ml anti-CD81 for 30 min prior to addition to plates; blocking antibodies were present during plating. Plates were kept at 37°C in a humidified incubator containing 10% CO_2 for 30 min. To remove unbound cells, wells were then filled with PBS and the plates were inverted in a tank of PBS and allowed to gently shake for 15 min. Excess PBS was absorbed from the wells by inverting plates on paper towels. Bound cells were fixed in 3% paraformaldehyde/PBS, then stained with 0.5% crystal violet in 20% methanol/80% H_2O. Wells were washed with water to remove excess dye, then cells were solubilized in 1% SDS and the amount of dye was quantified using a Molecular Devices plate reader set to absorb at 595 nm. Bars represent the average and standard deviation of 4 replicates.

The adhesion assay which measures strength of adhesion is an adaptation of the assays described by Calof and Lander (Calof and Lander, 1991). Briefly, a sheet of polystyrene was cut to fit a 96-hole silicon gasket (BioRad). The wells of the assay plate were coated with a titration of Ln-1 or heat denatured BSA, diluted in HBSS and incubated at 4°C for 4 h, then washed and blocked with 2% heat denatured BSA, at 4°C overnight. Transfected ES1 cells were pulsed with 10 μcuries ^{35}S-methionine for 1 h in 1 ml methionine free media then incubated overnight with the addition of 3 ml 10% CM. Labeled ES cells were introduced into the assay wells at a concentration of 50,000 cells/well. The plates were immediately centrifuged at 80 x G to synchonize cell contact with the substratum. The cells were allowed to bind for 30 min at 37°C, then the plates were flooded with warm PBS, sealed, inverted and centrifuged for 8 min at 80, 400, or 800 X G. The entire plate, still inverted, was submerged in cold PBS and then in fixative (3.7% formaldehyde / 5% sucrose / 0.1% Triton X 100 /PBS). After air-drying, the bound radioactivity, representing cell adhesion, was quantified on a Molecular Dynamics phosphorimager. Each point represents the average and standard deviation of 4 replicates.

Results

Murine ES1 cells expressing human integrin subunit α6A (ES6A) were obtained by transfection, as described in *Materials and Methods*. Surface expression of human α6A was verified by flow cytometry using two mAbs to human α6 (BQ16 and 2B7), which reacted equally well with ES6A cells (Fig. 2A). Furthermore, ES6A cells expressed heterodimers of human α6A associated with endogenous mouse β1, since immunoprecipitates of mouse β1 integrins contained α6A by Western blotting (Fig. 2D). Shown as control are flow cytometry analysis of ES1 cells transfected with vectors expressing either human α6B protein (ES6B) (Fig. 2B) or neo-resistance (ESneo) (Fig. 2C), and specific reactivity of the anti-α6A antiserum (Fig. 2E).

Inspection by light microscopy revealed that ES6A transfectants displayed morphological features unusual for undifferentiated ES cell cultures. ES6A cells formed loose colonies, with many individual cells appearing well separated (Fig. 3, top panel). Overall, morphology was reminiscent of motile cells, since many cytoplasmic protrusions or spikes were evident, recognizable as filopodia and lamellipodia (marked by arrowheads in Fig. 3). These morphological features were observed reproducibly in seven independent transfection experiments. They were not due to clonal variations, since ES6A transfectants were grown as bulk cultures that were FACS-selected for surface expression of human α6A (see Materials and Methods). Moreover, control ES6B (Fig. 3, middle panel) and ESneo (Fig. 3, bottom panel) transfectants displayed the same morphology as wild-type ES1 cells (which express endogenously only α6B, not α6A), i.e., cell cultures comprised mostly compact multicellular islands, with smooth borders, and rare isolated cells (Fig. 3, middle and bottom panels). These results indicated that expression of transfected α6A was likely responsible for the morphological changes of ES6A cell cultures.

Since the morphology of ES6A cells is reminiscent of motile cells, we performed haptotactic migration assays through porous membranes coated with purified ECM molecules, using transwell chambers. In these assays, ES6A cells exhibited dose-dependent high levels of migration on purified Ln-1 and Fn (Fig. 4A and 4B,respectively). Migration appeared to be dependent upon the presence of α6A, because control transfectants ES6B and ESneo did not migrate signifiacnatly (Fig. 4A and 4B). Furthermore, migration was observed on Ln-5 and migration on each of the purified matrices was blocked by GoH3, a function-blocking monoclonal antibody to both mouse and human α6 (Fig. 5).

Inhibition of migration on Ln-1 by GoH3 was expected, since α6β1 is a receptor for Ln-1 in most cell types studied (Hynes, 1992). However, in these cells, α6β1 is not required as a receptor for Ln-5, and there is no published evidence indicating that α6β1 may interact with Fn (Hynes, 1992). Therefore, we analyzed the role α6β1 plays in ES1 cell adhesion on Ln-5, Fn and Ln-1, i.e., matrices onto which migration is blocked by GoH3. In standard adhesion assays, ES6A, ES6B and ESneo cells adhered readily to Ln-1, Ln-5 or Fn (Fig. 6). However, only adhesion to Ln-1 was inhibited by the anti-α6 antibody GoH3 (Fig.6). Thus, in ES1 cells, α6β1 integrin is required as an adhesive receptor for Ln-1, but not for Ln-5 or Fn.

In the case of Ln-1, it was then possible to address the issue as to whether transfected α6A induced migration by changes in short-term cell-substratum adhesion strength, as recently proposed by Pelletier et al. (Pelletier et al., 1996) and Palecek et al. (Palecek et al., 1997). To characterize adhesion strength of ES6A cells on Ln-1, cells were tested in a centrifugal detachment assay. Fig. 7 shows that the number of cells adhering to Ln-1 decreased with increasing g force, but that the decrease was the same for ES6A, ES6B, and ESneo cells. We also tested ES6A cells for resistance to detachment under flow conditions (Savage et al., 1996) and found no differences from control ES6B and ESneo (data not shown). These data do not

support the idea that transfected human α6A alters adhesion strength to substratum as a mechanism for inducing migration.

On Ln-5 and Fn, migration was also unlikely to be induced by changes in adhesion strength, since α6Aβ1 apparently is not required as an adhesive receptor for these matrices. However, adhesion assays were performed over a shorter time than migration assays. To investigate whether α6Aβ1 may contribute to adhesion to those substrates over a longer period of time, we took advantage of the unique morphology of ES6A cells (Fig. 8A-F). If cells were pretreated with GoH3 and then plated on Fn, the extended cytoplasmic processes (Fig. 8A, arrowheads) were retracted completely. These experiments were performed for time periods matching the migration assay. After 18h, the GoH3-induced retraction of cytoplasmic processes persisted, even though cells were not detached (Fig. 8D). This correlated well with the block in migration (Fig. 5), and further suggested that transfected α6A induces ES6A migration and morphological changes on Ln-5 and Fn , but is not required for adhesion on these substrates.

It was still possible that ES1 cells deposited Ln-1, or a similar α6β1 adhesive ligand, over the course of the migration assay, and that such an adhesive ligand was responsible for the α6Aβ1-induced migration on Ln-5 or Fn. Migration assays were performed on filters saturated with purified matrices and excess ovalbumin to prevent this. Nonetheless, we tested migration in the presence of monensin, a broad inhibitor of secretion. Monensin was toxic to cells, in a dose-dependent manner. Therefore, decreases in migration activity may be expected, due to cytotoxicity. Since migration on Ln-1 should not require matrix secretion, we took migration levels on Ln-1 as a benchmark for maximum possible migration, at any given concentration of monensin. Levels of migration on Ln-5 or Fn were not lower than Ln-1 at any monensin concentration (Fig. 9), and at all points, migration was fully inhibitable by GoH3 (not shown). These results suggested that induction of migration by α6Aβ1 on Ln-5 or Fn did not occur via a deposited adhesive ligand.

To validate this point further, we used function-blocking antibodies to either Ln-1 or Ln-5 in migration assays. An anti-Ln-1 antiserum blocked migration on plated Ln-1, but did not affect ES6A migration on Fn or Ln-5 (Fig. 10A). CM6, a rat-specific anti-Ln 5 mAb, completely blocked migration on Ln-5 but had no effect on Ln-1 or Fn (Fig. 10B). These results demonstrated further that α6Aβ1-induced migration was not due to adhesive ECM deposited by ES6A cells.

Based on these data, α6Aβ1 induced changes in morphology and migration in ES6A cells without having to engage an ECM ligand. On the other hand, GoH3, a function-blocking anti-α6 antibody, did inhibit both morphological changes and migration, raising the possibility that, to cause these effects, α6Aβ1 was involved in molecular interactions that were interrupted by mAb GoH3. To investigate this possibility, we took advantage of the fact that antibody-mediated clustering of integrins can stimulate integrin-initiated functions, e.g., downstream signaling (Pelletier et al., 1992; Schwartz et al., 1995). After GoH3 was allowed to bind ES6A cells, goat anti-rat immunoglobulin antibody was added to cross-link the bound GoH3, hence clustering the GoH3-bound α6β1. In the case of Ln-1, which required α6β1 for ES1 cell adhesion (see Fig. 5), cross-linked GoH3 inhibited migration to the same extent as GoH3 alone (Fig. 11). Remarkably, though, on Ln-5 and Fn, cross-linking reversed the inhibition of migration by GoH3 (Fig. 11). Similarly, the morphology of ES6A cells plated on Fn was modulated by cross-linking GoH3: whereas GoH3 alone inhibited the extension of cytoplasmic processes characteristic of ES6A morphology (Fig. 8D), cross-linking GoH3 reversed this inhibition and re-established the motile-like morphology of ES6A cells (Fig. 8G). Thus, on substrates that do not require α6β1 for adhesion (i.e., Ln-5 and Fn), the changes in motility state of ES cells induced by α6Aβ1 may be triggered by the clustering of this integrin.

To explore further these molecular mechanisms, we tested the effects of antibodies to CD81 on ES cell adhesion and migration. CD81, a tetraspan protein, was recently shown to physically associate with several integrins, including α6Aβ1 (Hemler et al., 1996; Berditchevski et al., 1996; Rubinstein et al., 1996). By flow cytometry with the anti-CD81 antibody 2F7, ES cells were positive for surface CD81 (Fig. 12). In adhesion assays, anti-CD81 had no effect on the adhesion of the transfectants to Ln-1 or Fn (Fig. 13). In contrast, in migration assays, anti-CD81 strongly inhibited ES6A migration on Ln-1 and Fn (Fig. 14), while an isotype matched immunoglobulin control had no effect. These results indicate that the cell motility induced by α6Aβ1 may occur via a mechanism involving the integrin-associated protein CD81.

To investigate the possibility that the presence of only the α6A tail was directly inducing migration, chimeric molecules consisting of the CD8 extracellular domain and the cytoplasmic domain of either α6A or α6B, and as control, CD8 alone (Gaietta et al., 1994) were transfected into ES1 cells. Fig. 15 shows FACS analysis of the transfectants. The CD8/6A,CD8/6B, and CD8 transfectants, as well as ES1 cells, adhere similarly to Ln-1 and Fn (Data not shown). The CD8/6A and CD8/6B cells migrate better on Ln-1 and Fn than the CD8 control cells (Fig. 16). In light of the fact that clustering the α6A integrin with GoH3 also induces migration, we tested if clustering the chimeric molecule affected migration. After incubation with anti-CD8 antibody, CD8/6A and CD8/6B cells migrate 2-3 fold more than untreated cells (Fig. 16). Finally, this migration is dependent on CD81, as incubation with anti-CD81 blocks both constituitive migration as well as cluster-induced migration (Fig. 16).

Monoclonal antibodies were raised to human α6A. In hopes of identifying antibodies that would detect subtle conformational specificities of α6A, mAbs were raised to rat cells expressing human α6A (1NA4 cells). Mab's were islolated as described in Materials and Methods, using 4 levels of screening. After initial ELISA screens, a secondary ELISA was performed to eliminate clones that reacted to "background" rat proteins. FACS analysis was performed as a tertiary

screen, comparing parental RBL cells to 1NA4 cells. A final screen was immunoprecipitation. Three mAb's were identified, 12.2, 24.9 and 22.2, as shown by FACS (Fig. 17). Using a cell line, RBL 6A, that only expresses α6A, it was determined that mAb's 12.2 and 24.9 are specific for α6A, while 22.2 reacts with at least β4 or the complex α6Aβ4 (Fig. 17). All mAb's immunoprecipitate α6Aβ4 from cell surface-biotinylated lysates (Fig. 18). Investigations are underway to determine if the mAb's block the function of α6Aβ4 or α6Aβ1 in adhesion, migration and hemidesmosome formation assays.

Discussion

The goal of this grant was to identify the functional differences between the alternatively expressed cytoplasmic domains of the laminin receptor integrin, α6. Because adequate progress was not made towards a generating an α6-nulll cell line in which to perform the functional analysis, we chose to analyze ectopic expression of α6A in ES cells that endogenously expressed α6B. As a result, we showed that transfecting the α6A integrin isoform in ES cells induced both migration and a change in morphology on the ECM substrates Ln-1, Ln-5 and Fn. An intriguing aspect was that α6A did not contribute detectably to adhesion on Fn or Ln-5, in our assays. Therefore, α6A appeared to have a generalized effect on the motility state of ES1 cells, without having to engage with the ECM substrate onto which cells migrate.

Our initial observation was that α6A expression changed the morphology of ES1 cells, by promoting filopodia and lamellipodia extension, and disrupting the compact appearance of cell colonies. The relationship between morphology and motility has not been fully characterized in ES cells. Similar observations have been noted in other systems. For example, F9 teratocarcinoma induced to differentiate from stem cell to parietal endoderm with retinoic acid, show changes in cell morphology concomitant with induction of the α6A isoform (Jiang and Grabel, 1995). These data are consistent with our transfection system which shows an induction in shape change as a result of α6A expression. In our experiments, ES6A morphology correlated well with increased migration in transwell assays. Since our transfectants were kept in the presence of LIF, which prevents differentiation, α6A-induced morphological changes most likely occurred in the absence of differentiation. Both the increased motility and the morphological changes suggest a down-regulation of cell-cell adhesive contacts in ES6A cells. It will be interesting to determine whether α6Aβ1 has any effect on the affinity of, for example, cadherin interactions. Another line of evidence suggested a causal relationship between α6A and morphological changes: on Fn, addition of GoH3 (a function-blocking anti-α6 antibody) reversed the morphology of ES6A cells to that of wild-type ES1 cells.

One proposed mechanism for induction of migration considers a purely mechanical explanation (Lauffenburger and Horwitz, 1996). This theory proposes that cell migration depends upon two crucial parameters, intracellular motile force and receptor/ligand adhesive strength. An optimal ratio between these two parameters would be required for cells to locomote. Recently we reported that activation of the integrin αvβ3 also alters the migratory behavior of cells, and may do so by altering the strength of the integrin/ligand interaction (Huttenlocher et al., 1996). However, we tested this possibility in our system and found no differences in strength of adhesion to Ln-1 induced by α6A expression by two independent assays. Therefore, our data could not be explained satisfactorily on the basis of current theories on the mechanical aspects of migration, and suggested additional mechanisms for regulating cell migration.

Our results implicate the cytoplasmic domain of α6 as a regulatory site for migration. Because ES cells endogenously express the α6B isoform, but do not display the migratory phenotype of ES6A cells. Furthermore, transfection of human α6B, as an additional control, did not induce the migratory phenotype. Structural differences between α6A and α6B are limited to the cytoplasmic domains. It remains to be determined how these structural differences relate to the induction of migration by α6A. There has been a report of differences in tyrosine phosporylation of other molecules induced by ligating α6A vs. α6B in transfected macrophages (Shaw et al., 1995). However, the relationship between these phosphorylation events and migration has not been defined. Additionally, it has not been established whether the two serines that exist in the α6A tail, which can be phospohorylated, play a role in cell migration (Hogervorst et al., 1993; Shaw and Mercurio, 1993).

The following four independent lines of evidence eliminated the possibility that ES6A cells deposited an adhesive ligand responsible for the migration on Fn and Ln-5: i) anti-Ln-1 antiserum blocked migration on Ln-1, yet had no effect on migration on Fn or Ln-5; ii)

similarly, an anti-Ln-5 mAb completely blocked migration on Ln-5, yet had no effect on Ln-1 or Fn; iii) vitronectin, collagen IV, or BSA did not support migration (SZD, data not shown); iv) treatment with monensin, an inhibitor of secretion, did not block migration on Fn or Ln-5 any more than the toxic effect observed for ES6A cells migrating on Ln-1. Thus far, on all substrates to which ES cells adhere, ES6A cells show enhanced migration irrespective of whether α6 is involved directly with adhesion on that substrate.

In further investigations on the induction of migration by α6A, we found that GoH3 completely blocked migration on Ln-1, Ln-5 and Fn. However, cross-linking of GoH3 with a secondary antibody restored migration on Ln-5 and Fn, i.e., those substrates on which α6β1 was not required as a receptor for adhesion. This suggests a model whereby expression of α6A is not by itself sufficient to induce migration. Rather, α6A may have to become involved in molecular interactions that can be inhibited by GoH3. It is intriguing, and perhaps unexpected that clustering of GoH3 reversed this inhibition. Several explanations are possible, since bound GoH3 antibody may interfere with many conceivable mechanisms (e.g., receptor recycling rate) that could be reinstated by clustering. One explanation we favor is that clustering mimics the binding of α6β1 to some counter-receptor that signals the cells to become more motile, irrespective of the adhesive ligand onto which migration occurs. If this were the case, it might be expected that distinct structural determinants on the α6A subunit may underlie adhesive versus migratory functions. Interestingly, Chan and collaborators recently reported a monoclonal antibody to α6β1 that inhibits migration but not adhesion (Hangan et al., 1997). It will be interesting to see whether the determinant recognized by this antibody is involved in the induction of motility we describe here.

Our data support a correlation between migration and the marked increase in long filopodia in ES6A cells. In every case, these two parameters where either induced or inhibited concurrently. For instance, GoH3 alone both inhibited migration and reversed the morphological phenotype.

Cross-linking of α6Aβ1 with GoH3 restored both migration and the filopodia. In the haptotactic migration assay, cells presumably extend processes through the pores to detect the matrix on the underside of the filter. Thus, the extension of long filopodia in ES6A cells may be directly responsible for increased migration. Defining the molecular basis for these effects is an area for future investigations.

One important consequence of our data is that inhibition of migration by anti-α6 antibodies no longer signifies unequivocally that cells are migrating on adhesive ligands for α6β1. To our knowledge, this is the first time that a function-perturbing antibody, such as GoH3, was shown to block migration without blocking adhesion. In light of these results, reevaluation of the role of some function-blocking antibodies may be required.

To investigate molecular mechanisms whereby α6β1 can influence migration mediated by other integrins, we explored the possible role of integrin associated proteins, particularly transmembrane ones. Several members of the tetraspan superfamily, e.g., CD9, CD63, CD81 and CD82, were convincingly documented to form molecular complexes with integrins at the cell surface. Tetraspan proteins are expressed in many cell types, and comprise two short cytoplasmic domains (the amino and carboxy terminus), two unequal extracellular loops and four membrane spanning segments. Though tetraspan proteins have been implicated in many cellular activities, including adhesion, migration and proliferation, their exact function is unknown (Hemler et al., 1996). Among the possibilities, they were proposed to act as networking pieces for cell surface proteins, such as signal transducers associated to G proteins, or as ion channels. Any of these functions are conceivable in their part as integrin-associated proteins.

Recently, an antibody to the tetraspan protein CD81, 2F7, was described to inhibit T cell maturation in fetal thymus organ cultures (Boismenu et al., 1996). We show here that this same antibody inhibits motility induced by α6Aβ1. These results are consistent with the recent

proposals that tetraspan proteins may influence integrin-mediated migration (Shaw et al., 1995), primarily based upon data showing the enhanced motility of B cell lines transfected with CD9, the inhibition of haptotactic migration with anti-CD9 antibody, and the identification of a tetraspan as a metastasis suppressor gene (Dong et al., 1995). However, to our knowledge, this is the first report that anti-CD81 antibodies inhibit cell motility.

It is worth pointing out that, in our system, anti-CD81 antibodies blocked the motility induced by an integrin not involved in adhesion to the migratory substrate. Furthermore , the anti-CD81 antibody had no effect on adhesion of ES cells. Therefore, our results are consistent with the prevalent view that tetraspan proteins may not be involved in modulating integrin-mediated adhesion (Hemler et al., 1996). Additionally, to show specificity to the α6 integrin, CD81 did not block migration on Collagen IV by embryonic fibroblasts, where migration is mediated by the integrin α1β1 (data not shown). We propose a model whereby, in ES6A cells, the physical interaction between α6Aβ1 and CD81 triggers signaling events that result in upregulation of motility and modulation of function of other integrins engaged with migratory substrate. It is also possible that CD81 plays an alternative or additional role at the level of the migratory integrins themselves. Further studies with ES6A cells, aimed at establishing the molecular interactions between CD81 and integrins, as well as their functional consequences, will help clarify the validity of this model.

To further dissect the role of the cytoplasmic domains of α6 in migration, chimeric surface molecules consisting of the extracellular domain of CD8 fused to the α6A or α6B cytoplasmic tails were expressed in ES cells. Both molecules induced migration as compared to the control CD8 alone transfectant. There may be several explanations for this result, which is in contrast to the findings that the full-length α6B integrin does not support significant migration. A likely hypothesis is based on the idea that the beta chains of the integrin receptor regulate the function of the integrin. It is possible that in the α6β1 heterodimer, the beta chain maintains the α6B tail

in a conformation that does not allow for interaction with molecules necessary for migration. Additionally, in the CD8 chimera, the α6A and α6B tails are allowed to dimerize, since CD8 exists as a dimer. This may keep the α6B tails in a conformation that promotes migration. CD81 remains an important component in the induction of migration. mAb's to CD81 block the chimera-induced migration as well as the increase in migration resulting from crosslinking of CD8.

Finally, progress has been made towards Aim 4. Three monoclonal antibodies have been identified against human α6A and α6Aβ4. Work will continue on characterizing the effect of these antibodies on adhesion, migration, and the formation of hemidesmosomes. It is hoped that the mAb's may detect an activation epitope on the integrins or perhaps block the function of the integrin receptors.

Conclusions

We showed that transfecting the α6A integrin isoform in ES cells induced both migration and a change in morphology on the ECM substrates Ln-1, Ln-5 and Fn. An intriguing aspect was that α6A did not contribute detectably to adhesion on Fn or Ln-5, in our assays. Therefore, α6A appeared to have a generalized effect on the motility state of ES1 cells, without having to engage with the ECM substrate onto which cells migrate. Migration was also dependent on the tetraspanin family member, CD81. This work significantly expanded our understanding of α6 integrin-mediated migration. In a broader sense, this work may increase our understanding of metastasis in cancer.

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Fig. 1. **Alternate cytoplasmic tails of the α6 integrin subunit.** Shown is the single letter amino acid sequence of the α6A and α6B cytoplasmic tails beginning at amino acid 988. The extracellular domains are identical from amino acid 1 through 994, afterwhich the sequences are completely divergent. An arrowhead marks the point where the sequences begin to differ.

Fig. 2. **ES6A transfectant cells express human α6A on the cell surface, complexed with endogenous mouse β1.** Expression of full length human α6A or α6B cDNA was analyzed by FACs analysis of A.) ES6A, B.) ES6B, and C.) ESneo cells, using FITC-secondary antibody alone (black fill), mAb BQ16 and 2B7 (anti-human α6) (dashed/dotted lines) and EA1 (anti-mouse and human α6) (solid line). Cells were analyzed on a FACscan machine (Becton Dickinson). D.) Cell lysates were subjected to immunoprecipitation using sepharose beads coupled to the anti-β1 mAb, 9EG7. Beads were eluted in non-reducing (odd numbered lanes) or reducing (even numbered lanes) sample buffer, separated on SDS-PAGE and transferred to PVDF. Whole lysate (lanes 1,2,5,6) was electrophoresed along with the anti-β1 immunoprecipitations. Lanes 1-4 were probed with anti-α6A antibody and lanes 5-8 were probed with anti-α6B antibody. E.) As control to show specificity of the anti-peptide antibodies, whole lysates from ES6A (lanes 1,3) and ES6B (lanes 2,4) were separated under non-reducing conditions. Lanes 1 and 2 were probed with anti-α6A antibody and lanes 3 and 4 were probed with anti-α6B antibody. In both cases (D and E) the arrow indicates a band corresponding to non-reduced α6 polypeptide detected at approximately 140kd.

Fig. 3. **The periphery of ES6A cells, but not ES6B or ESneo cells, exhibits lamellipodia and filopodia.** Cells were grown on Ln-1 coated glass coverslips overnight, washed with PBS, fixed in 2.5% paraformaldehyde/PBS, and mounted in Immunofluore (ICN, Costa Mesa, CA). Cells were visualized in phase contrast using a Zeiss Axiovert microscope and photographed using

TMAX 400 ASA film. Top panel (ES6A, arrowheads indicate lamellipodia and filopodia), middle panel (ES6B), bottom panel (ESneo). The scale bar is 20 microns.

Fig. 4. **ES6A cells migrate on Ln-1 and Fn.** Transwell filters were coated for 4 h at 37°C with varying concentrations of Ln-1 or Fn. ES6A, ES6B or ESneo cells (1 x 10^4 cells/filter) were plated on the uncoated side. Cells were maintained at 37°C for 18 h, then filters were fixed and stained. To quantify migration, stained cells were counted in four fields (using a 40x objective) from each of two filters for each condition. Results of representative experiments are expressed as the mean number of cells counted in each field +/- the standard deviation..

Fig. 5. **ES6A cells migration on Ln-1, Fn and Ln-5 is blocked by anti-α6 mAb GoH3.** Transwell filters were coated for 4 h at 37°C with 40 μg/ml of Ln-1 or Fn or 10 μg/ml Ln-5. ES6A, ES6B or ESneo cells (1 x 10^4 cells/filter) were plated on the uncoated side. For blocking experiments, cells were incubated at room temperature with 10 μg/ml GoH3 antibody in migration medium for 30 min prior to plating on coated filters . Cells were maintained at 37°C for 18 h, then filters were fixed and stained. To quantify migration, stained cells were counted in four fields (using a 40x objective) from each of two filters for each condition. Results of representative experiments are expressed as the mean number of cells counted in each field +/- the standard deviation..

Fig. 6. **ES6A, ES6B, and ESneo cells adhere to Ln-1 in an α6-dependent manner, and to Fn and Ln-5 in an α6-independent manner.** Ln-1, Fn (both at 20 μg/ml) and Ln-5 (1μg/ml) were coated in 0.2 M carbonate buffer onto 96 well plates at 37°C for 4 h. Plates were washed and blocked for 1 h. For blocking with GoH3, cells were pre incubated with 10 μg/ml GoH3, then plated in the presence of antibody. Cells adhered for 30 min at 37°C, washed, fixed, and stained. Stained cells were solubilized with 1% SDS and absorbance was quantified at 595 nm.

Fig. 7. **ES6A cells do not differ in strength of adhesion to Ln-1.** The wells of the assay plate were coated with a titration of Ln-1 or heat denatured BSA, diluted in HBSS and incubated at 4°C for 4 h, then washed and blocked with 2% heat denatured BSA, at 4°C overnight. ^{35}S-methionine-labeled ES6A, ES6B or ESneo cells were plated (10,000 cells/well). The plates were immediately centrifuged at 80 x g to synchronize cell contact with the substratum. The cells were allowed to bind for 30 min at 37°C, then the plates were flooded with warm PBS, sealed, inverted and centrifuged for 8 min at 80, top panel, 400, middle panel, or 800, bottom panel, X g. The entire plate, still inverted, was submerged in cold PBS and then in fixative (3.7% formaldehyde / 5% sucrose / 0.1% Triton X 100 /PBS). After air-drying, the bound radioactivity, representing cell adhesion, was quantified on a Molecular Dynamics phosphorimager.

Fig. 8. **ES6A filopodia and lamellipodia are blocked by anti-α6 antibody GoH3, and are restored by cross-linking GoH3 with secondary antibody.** Glass coverslips were coated for 30 min at 37°C with 40 μg/ml of Fn. ES6A, ES6B or ESneo cells (1 x 10^4 cells/coverslip) were plated. For antibody treatments, cells were incubated at room temperature with 10 μg/ml GoH3 or 10 μg/ml goat anti-rat affinity-pure antibody in migration medium for 15 min. For cross-linking, 10 μg/ml goat anti-rat affinity-pure antibody was added for an additional 15 min to cells that had bound GoH3. No antibodies were removed before plating on the coverslips. Cells were maintained at 37°C for 18 h, then washed with PBS, fixed in 2.5 % paraformaldehyde/PBS, and mounted in Immunofluore. Cells were visualized in phase contrast using a Zeiss Axiovert microscope and photographed using TMAX 400 ASA film. Panels A, D, G are ES6A cells: control, GoH3, and cross-linked GoH3, respectively; panels B, E, H are ES6B cells: control, GoH3, and cross-linked GoH3, respectively; and panels C, F, I are ESneo cells: control, GoH3, and cross-linked GoH3, respectively. The scale bar represents 20 microns.

Fig. 9. **Monensin does not abolish α6A-induced migration on Fn.** Transwell filters were coated for 4 h at 37°C with 40 μg/ml of Ln-1 or Fn or 10 μg/ml Ln-5. ES6A, ES6B or ESneo

cells (1 x 10^4 cells/filter) were plated on the uncoated side of filters in migration media, or migration media including 0.01, 0.1 or 1 μM monensin. In all cases, cells were maintained at 37°C for 18 h, then filters were fixed, stained and analyzed as previously described.

Fig. 10. **ES6A migration is blocked by anti-Ln-1 on anti-Ln-5 antibodies only on the corresponding ECM substrates.** Transwell filters were coated for 4 h at 37°C with 40 μg/ml of Ln-1 or Fn or 10 μg/ml Ln-5. ES6A, ES6B or ESneo cells (1 x 10^4 cells/filter) were plated on the uncoated side. For blocking experiments, cells were incubated at room temperature with a 1:100 dilution of anti-Ln-1 antibody in migration medium for 30 min prior to plating on coated filters (panel A). For anti-Ln-5 blocking experiments, ES6A cells were incubated at room temperature with 25 μg/ml CM6 or as control, 25 μg/ml MOPC in migration medium for 30 min prior to plating on coated filters. In all cases, cells were maintained at 37°C for 18 h, then filters were fixed, stained and analyzed as previously described.

Fig. 11. **Anti-α6 antibody GoH3 blocks migration on Fn and Ln-5, but cross-linking GoH3 with secondary antibody restores it.** Transwell filters were coated for 4 h at 37°C with 40 μg/ml of Ln-1 or Fn or 10 μg/ml Ln-5. ES6A, ES6B or ESneo cells (1 x 10^4 cells/filter) were plated on the uncoated side. For controls, cells were incubated at room temperature with 10 μg/ml GoH3 or 10 μg/ml goat anti-rat affinity-pure antibody in migration medium for 15 min. For cross-linking, 10 μg/ml goat anti-rat affinity-pure antibody was added for an additional 15 min to cells that had bound GoH3. No antibodies were removed before plating on the filters. In all cases, cells were maintained at 37°C for 18 h, then filters were fixed, stained and analyzed as previously described. ES6A (panel A), ES6B (panel B), ESneo (panel C).

Fig. 12. **ES cells express CD81.** Expression of CD81 was analyzed by FACs analysis of ES cells using FITC-secondary antibody alone (black fill) and mAb 2F7 (anti-CD81) (solid line). Cells were analyzed on a FACscan machine (Becton Dickinson).

Fig. 13. **Anti-CD81 antibody does not affect adhesion.** Ln-1 and Fn (both at 20 μg/ml) were coated in 0.2 M carbonate buffer onto 96 well plates at 37°C for 4 h. Plates were washed and blocked for 1 h. For blocking with anti-CD81, cells were pre incubated with 75 μg/ml anti-CD81, then plated in the presence of antibody. Cells adhered for 30 min at 37°C, washed, fixed, and stained. Stained cells were solubilized with 1% SDS and absorbance was quantified at 595 nm.

Fig. 14. **Anti-CD81 antibody blocks migration on Ln-1 and Fn .** Transwell filters were coated for 4 h at 37°C with 20 μg/ml of Ln-1 or Fn. ES6A, ES6B or ESneo cells (1 x 10^4 cells/filter) were plated on the uncoated side. Cells were incubated with 75 μg/ml hamster anti-CD81 (2F7) or for controls, cells were incubated with 2% glycerol in PBS (since 2F7 is supplied in 20% glycerol) or with 75 μg/ml hamster anti-TNP antibody, in migration medium for 30 min. No antibodies were removed before plating on the filters. In all cases, cells were maintained at 37°C for 18 h, then filters were fixed, stained and analyzed as previously described.

Fig. 15. **CD8/6A and CD8/6B are expressed in ES cells at similar level.** Expression of full length human CD8/6A or CD8/6B cDNA was analyzed by FACs analysis, using anti-human CD8 coupled to phycoerytherin. Cells were analyzed on a FACscan machine (Becton Dickenson).

Fig. 16. **Migration of CD8/6A and CD8/6B cells is increased by crosslinking CD8 and is dependent on CD81.** Transwell filters were coated for 4 h at 37°C with 20 μg/ml of Ln-1 (panel A) or Fn (panel B). CD8/6A, CD8/6Bcells, and CD8 cells (1 x 10^4 cells/filter) were plated on the uncoated side. Cells were incubated with 10 μg/ml anti-CD8, 75 μg/ml hamster anti-CD81 (2F7), anti-CD8 + anti-CD81, or for controls, cells were incubated with 2% glycerol in PBS (since 2F7 is supplied in 20% glycerol) in migration medium for 30 min. No antibodies were

removed before plating on the filters. In all cases, cells were maintained at 37°C for 18 h, then filters were fixed, stained and analyzed as previously described.

Fig. 17. **Three mAb's bind 1NA4 cells.** Expression of human α6Aβ4 was detected by new rat mAb's 12.2 (solid line), 24.9 (dotted line), and 22.2 (dashed line)and negative control, secondary alone (black fill). Cells were analyzed on a FACscan machine (Becton Dickenson).

Fig. 18. **mAb's 12.2, 24.9 and 22.2 all immunoprecipitate α6Aβ4 from 1NA4 cells.** Biotinylated 1NA4 and RBL cell lysates were subjected to immunoprecipitation using mAb 12.2 (panel A), 24.9 (panel B) or 22.2 (panel C) bound to Protein G Sepharose beads. Protiens were eluted in reducing (odd numbered lanes) or non-reducing (even numbered lanes) sample buffer, separated on SDS-PAGE and transferred to PVDF. Biotinylated proteins were detected by binding avindin-horseradish peroxidase and detected using ECL. Panel A: Lanes 1, 2, 7, 8, GoH3 mAb; lanes 3, 4, 9, 10; 12.2 mAb; lanes 5, 6, 11, 12, no mAb; lanes 1-6, 1NA4 lysate; lanes 7-12, negative control, RBL lysate. Panel B: Lanes 1, 2, no mAb; lanes 3,4,7,8, GoH3 mAb; lanes 5,6,9,10, 24.9 mAb; lanes 1-6, 1NA4 lysate; lanes 7-10, negative control, RBL lysate. Panel C: Lanes 1,2 7, 8, no mAb; lanes 3, 4, 9, 10; GoH3 mAb; lanes 5,6,9,10, 22.2 mAb; lanes 1-6, 1NA4 lysate; lanes 7-12, negative control, RBL lysate.

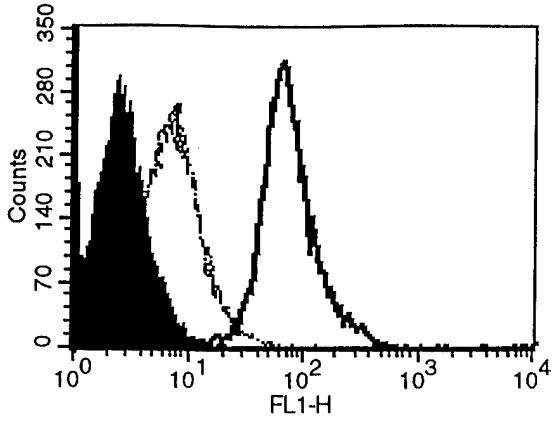
α6A

-988-KCGFFKRNKKDHYDATYHKAEIHAQPSDKERLTSDA-COOH

α6B

-988-KCGFFKRSRYDDSVPRYHAVRIRKEEREIKDEKYIDNLKKQWITKWNRNESYS-COOH

A.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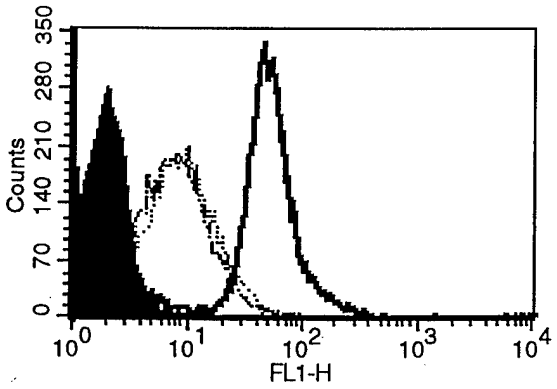
B.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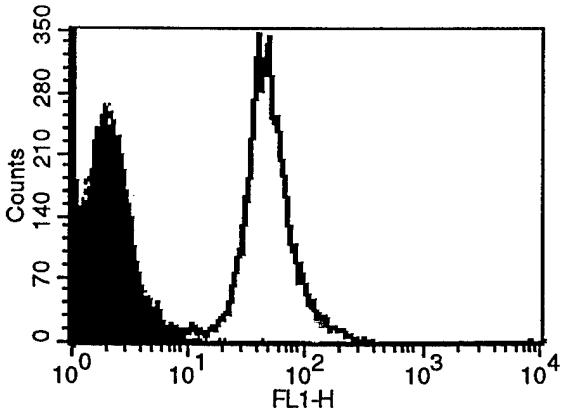
C.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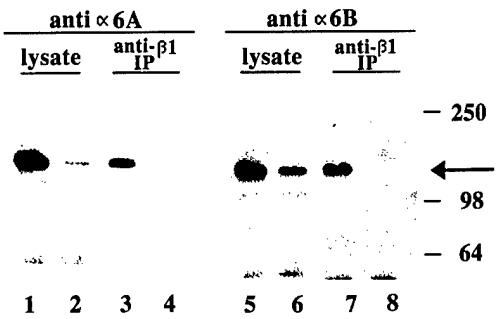
D.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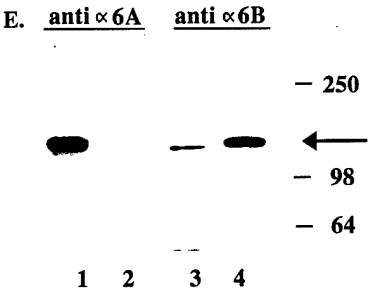
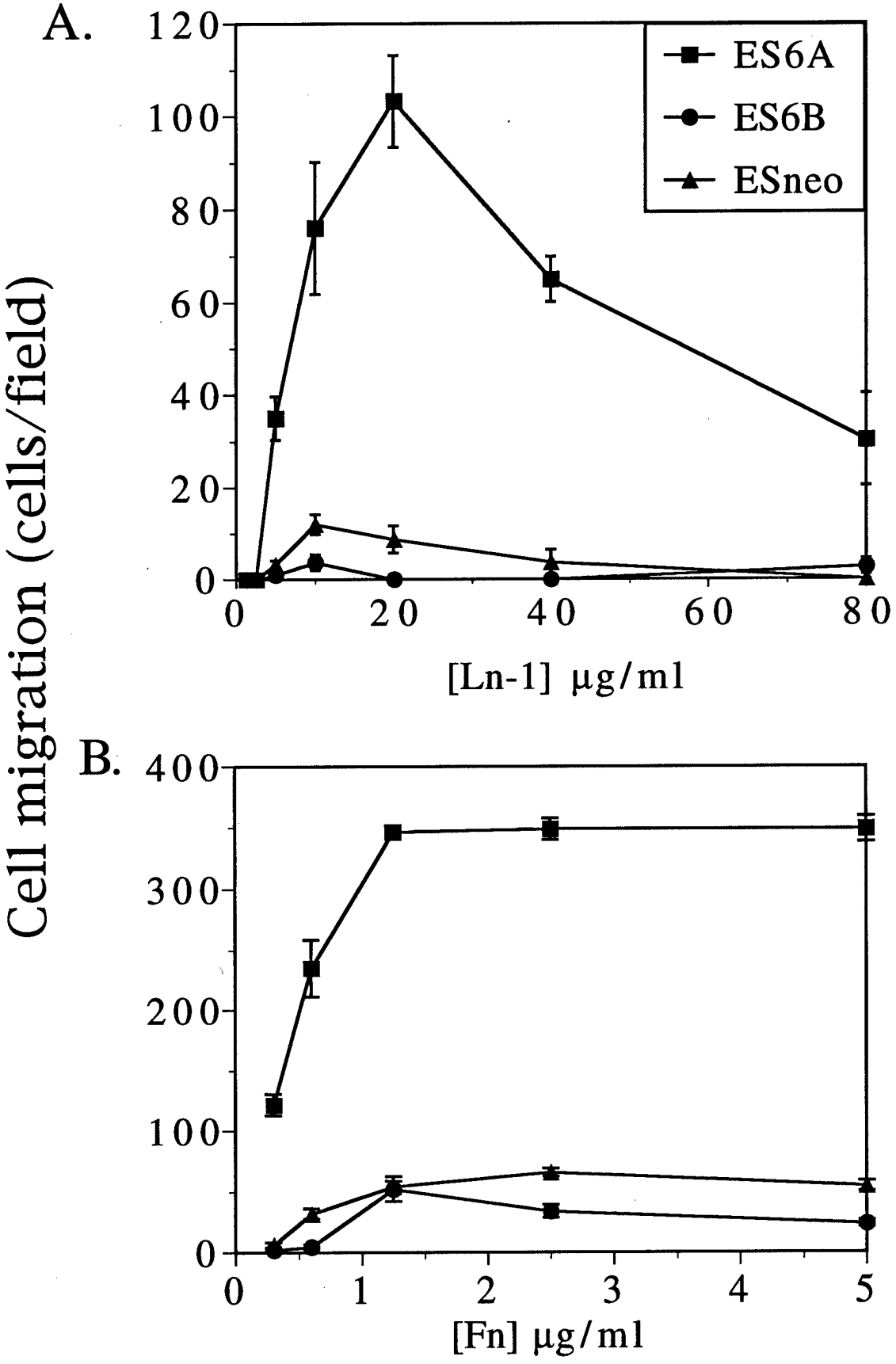
anti ∝6B
lysate
anti-β1 IP
lysate
anti-β1 IP
250
98
64
1 2 3 4 5 6 7 8

E.

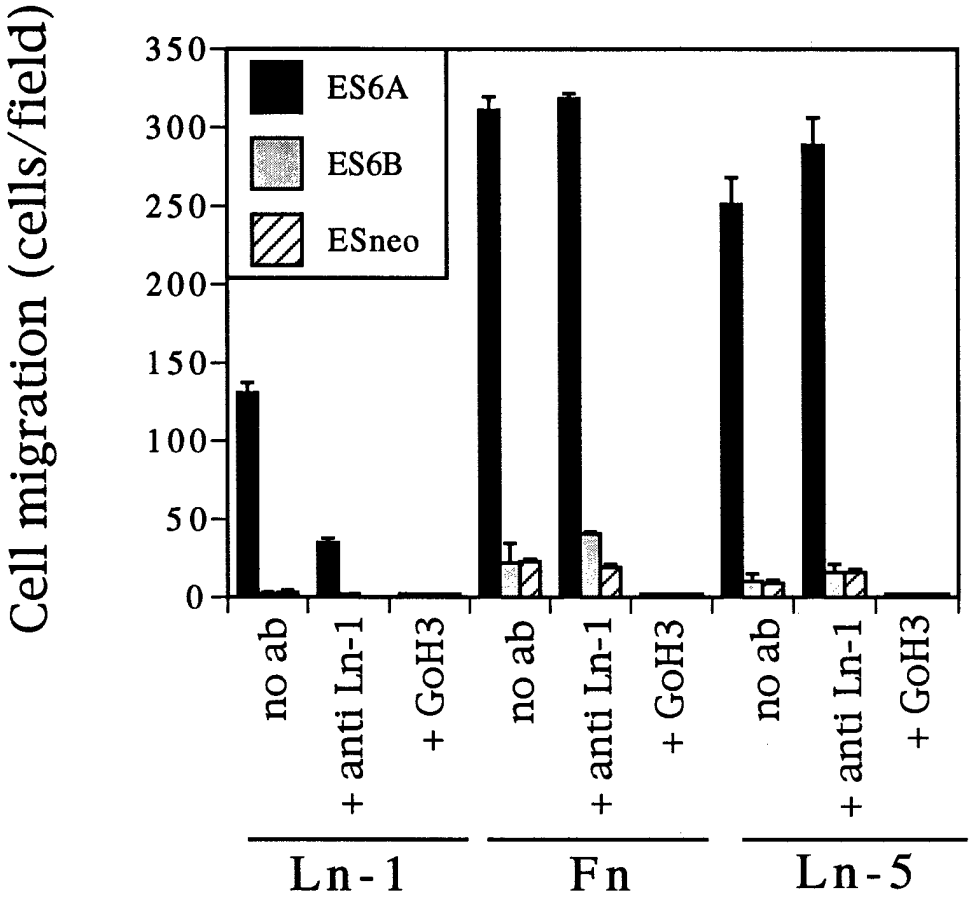

anti ∝6A
anti ∝6B
250
98
64
1 2 3 4

The periphery of ES6A cells exhibits lamellipodia and filopodia

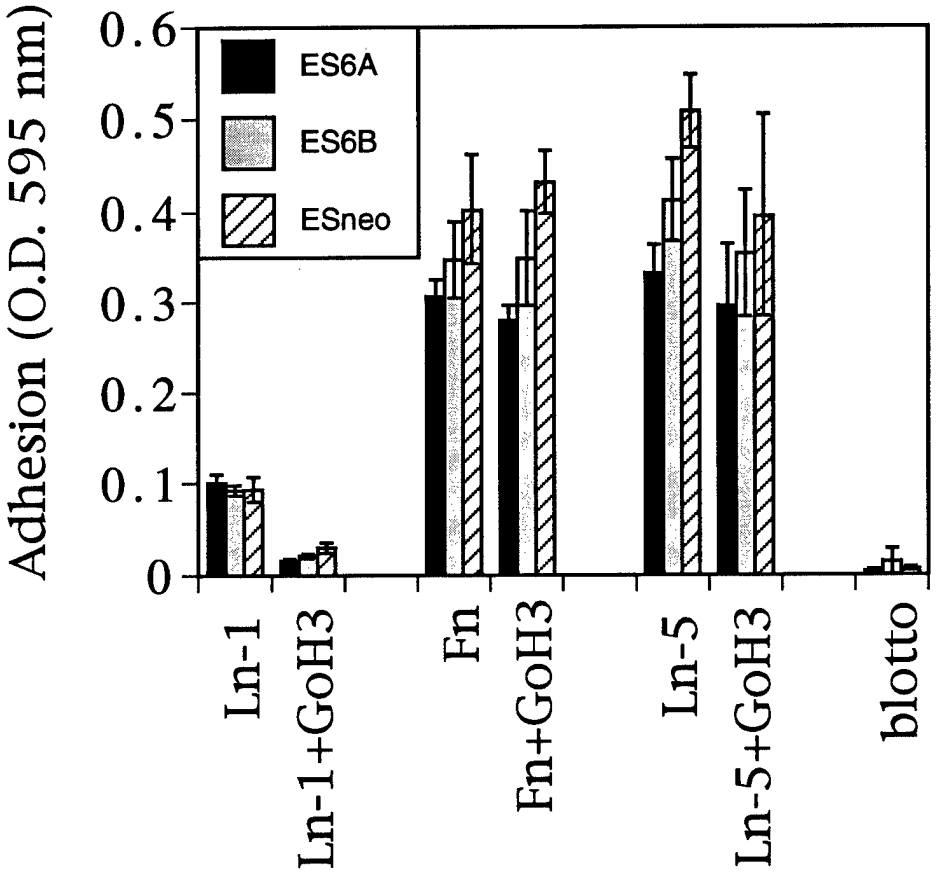
ES6A cells migrate on Ln-1 and Fn



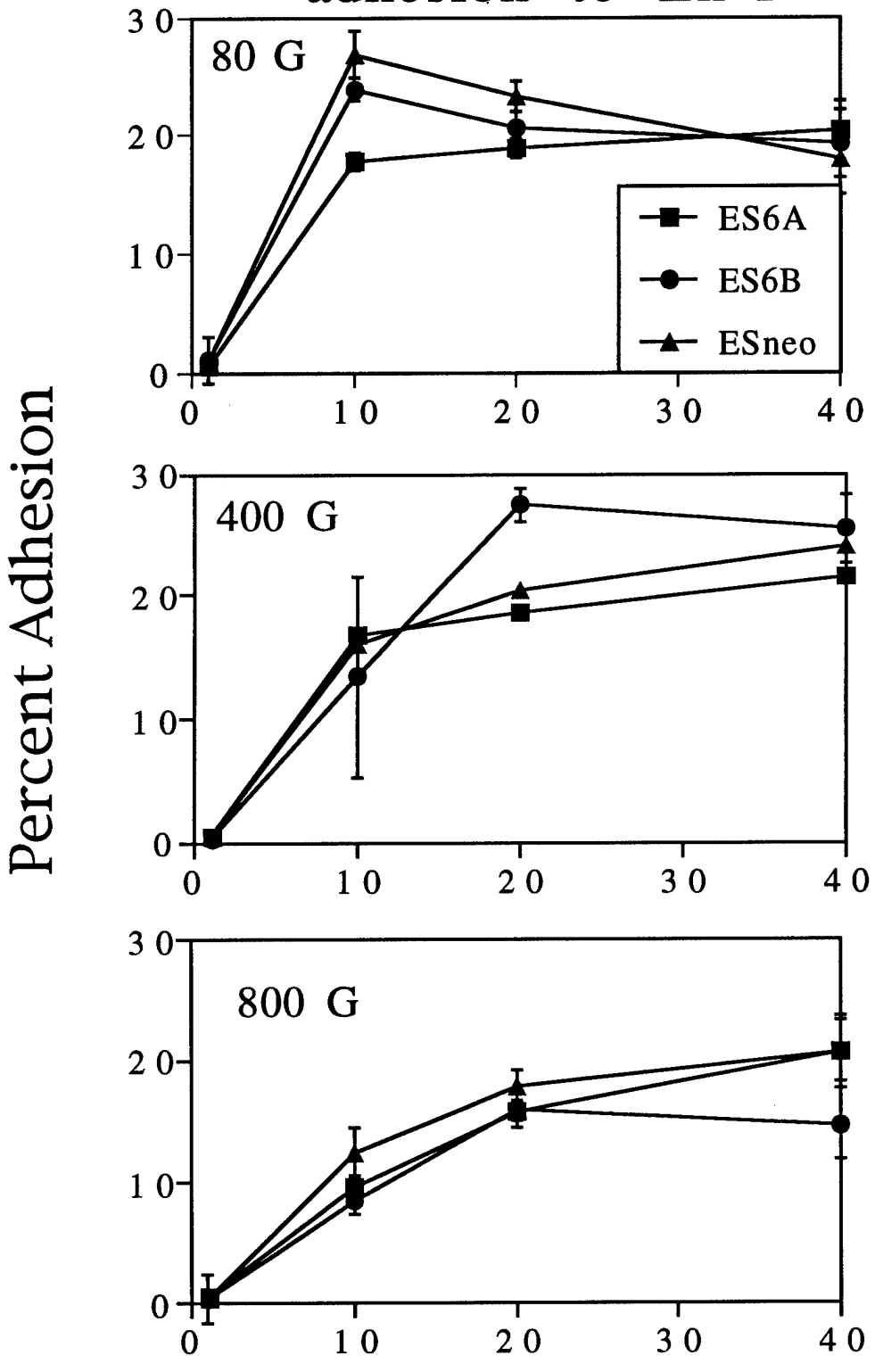
ES6A cell migration on Ln-1, Fn, and Ln-5 is blocked by anti-α6 mAb

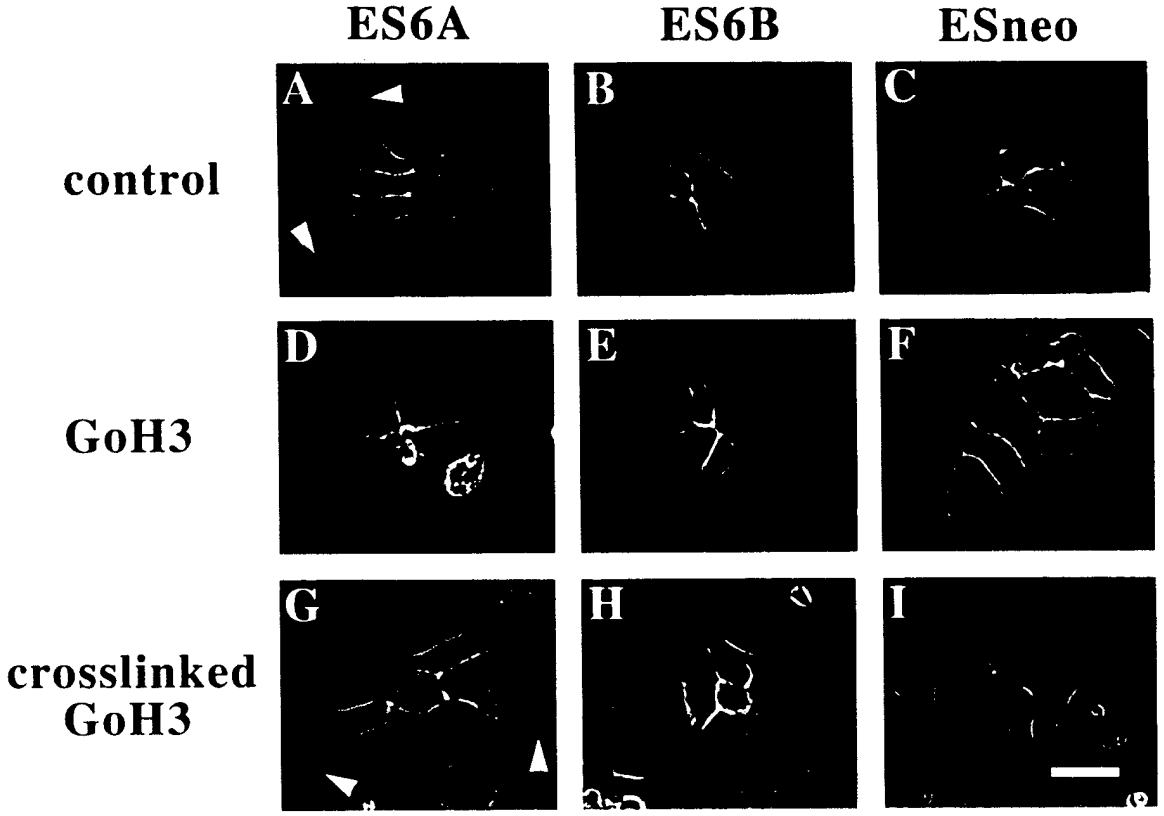


ES6A, ES6B, and ESneo cell-adhesion to Fn is α6-independent

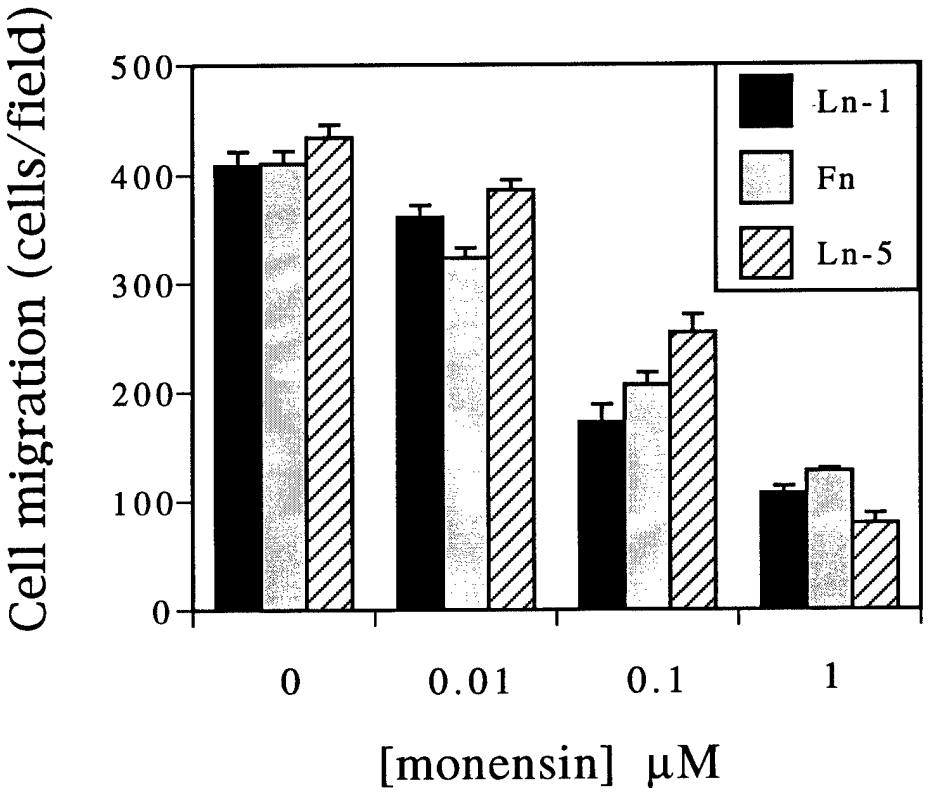


ES6A cells do not differ in strength of adhesion to Ln-1

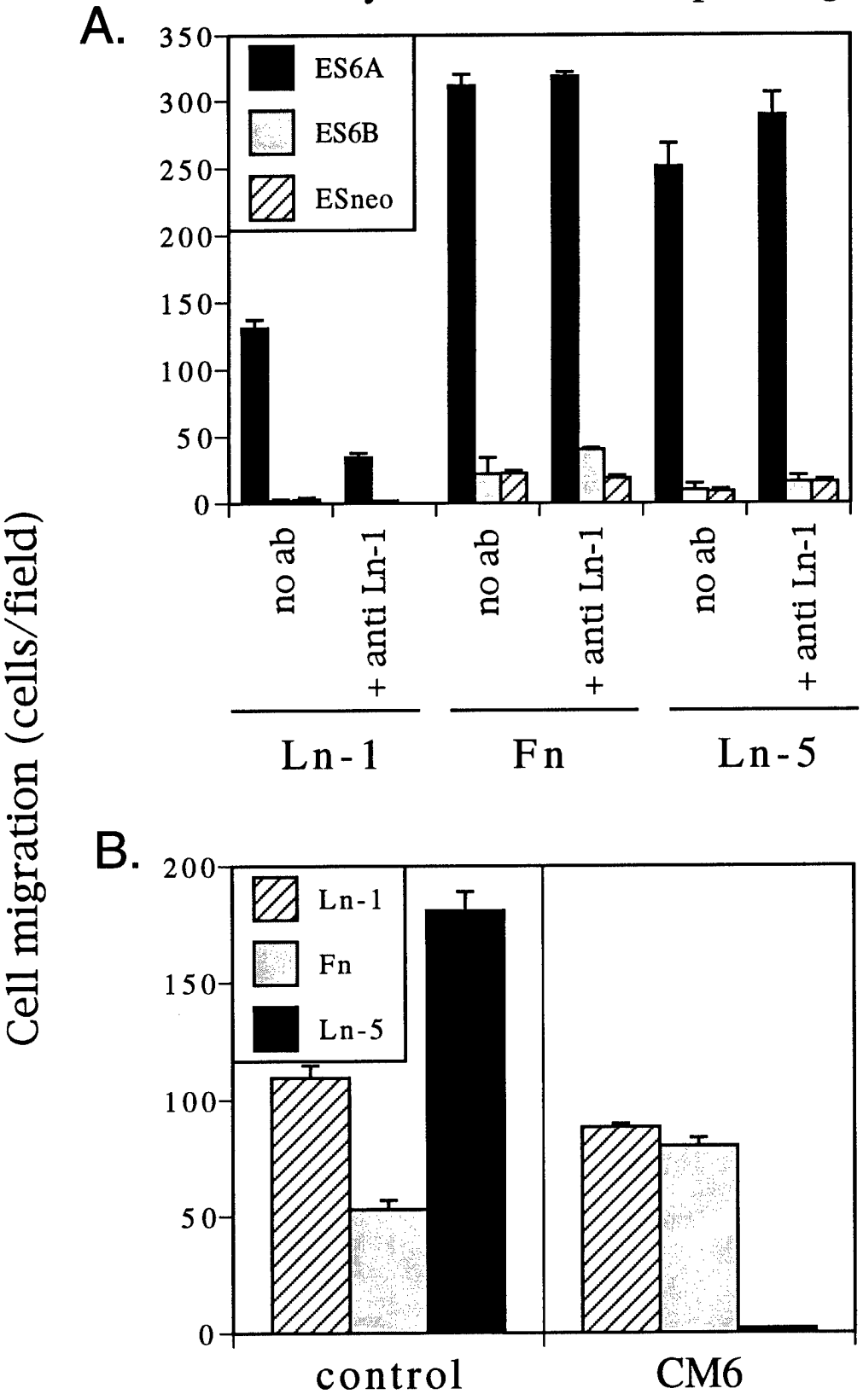



ES6A
ES6B
ESneo
control
GoH3
crosslinked GoH3
A
B
C
D
E
F
G
H
I

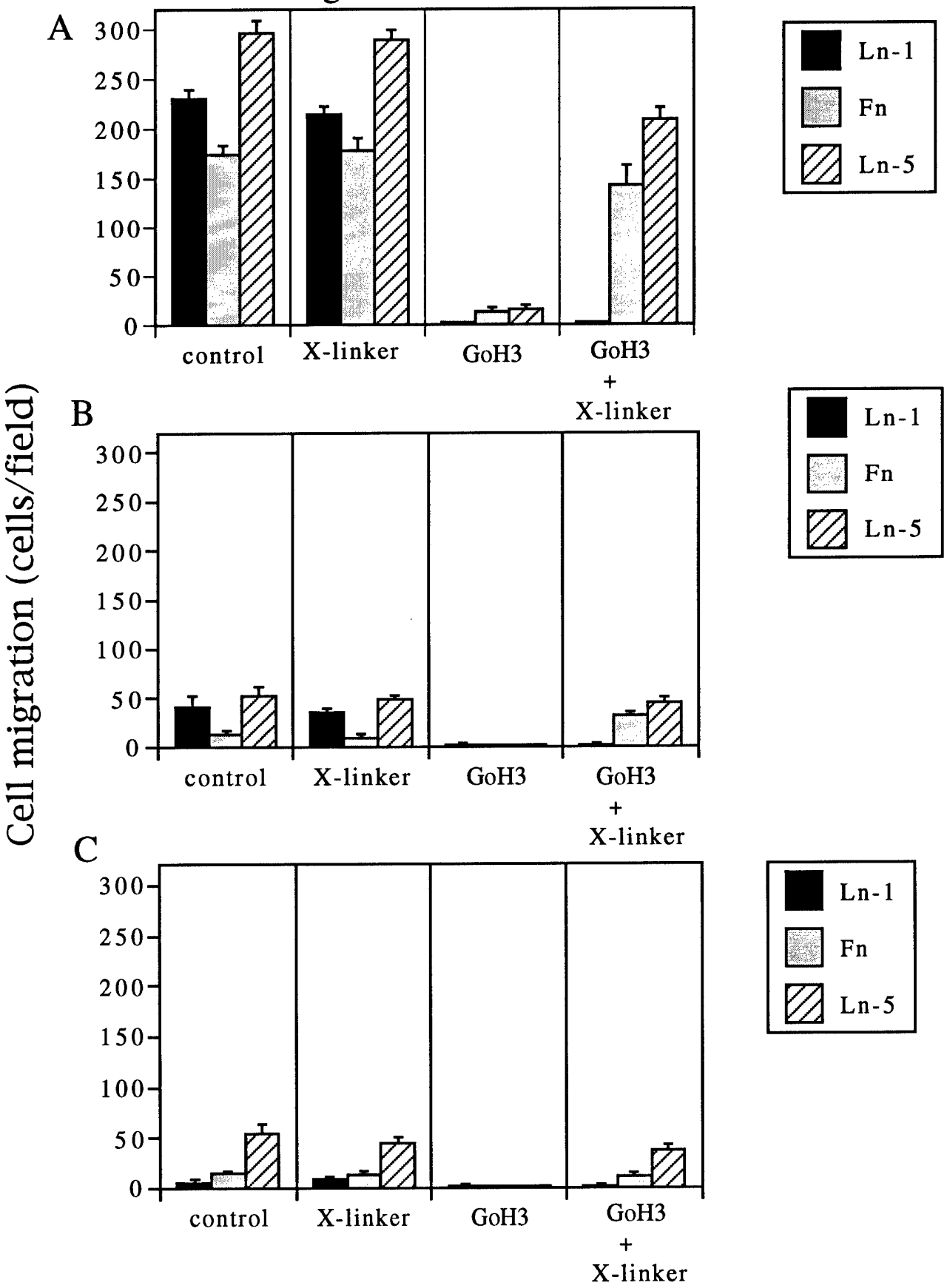
Monensin does not abolish α6A-induced migration on Fn or Ln-5

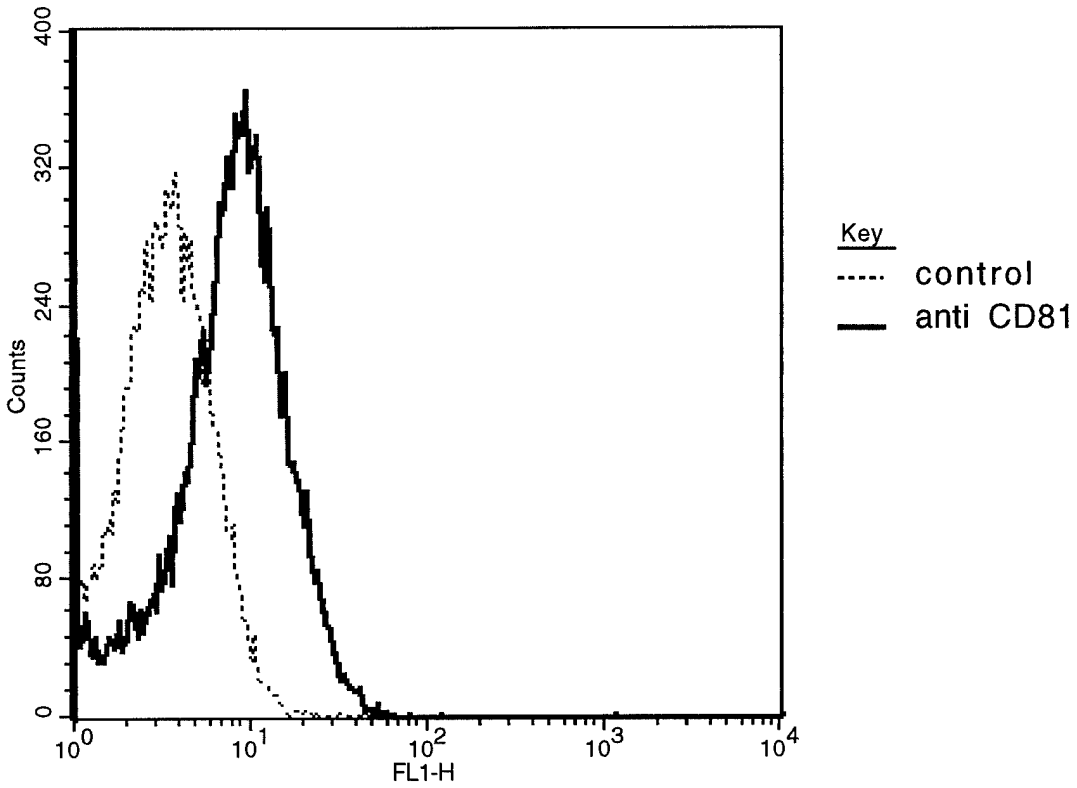


ES6A migration is blocked by anti-Ln-1 or anti-Ln-5 antibodies only on the corresponding matrices



X-linking with anti-α6 mAb restores mAb-blocked migration on Fn and Ln-5

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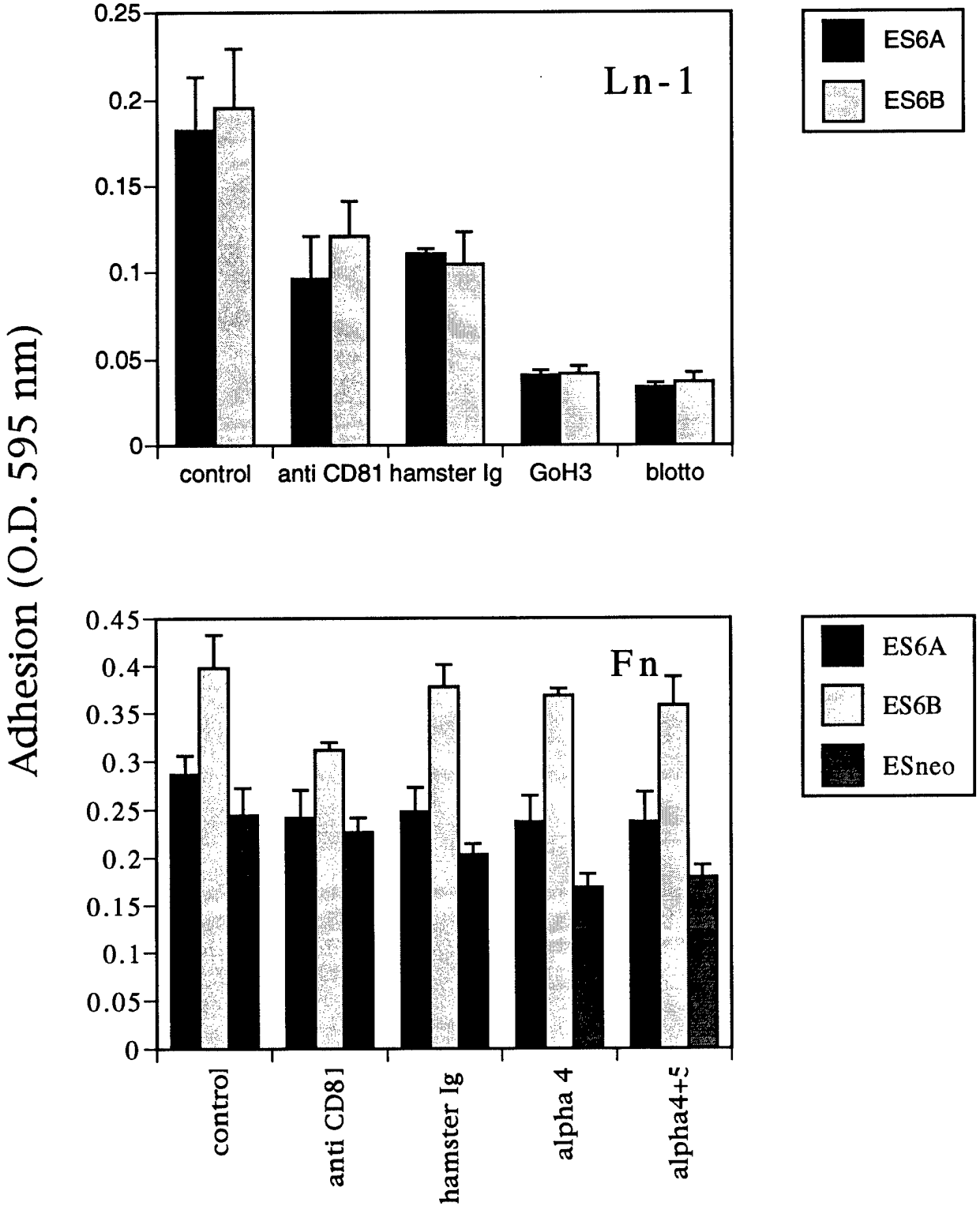
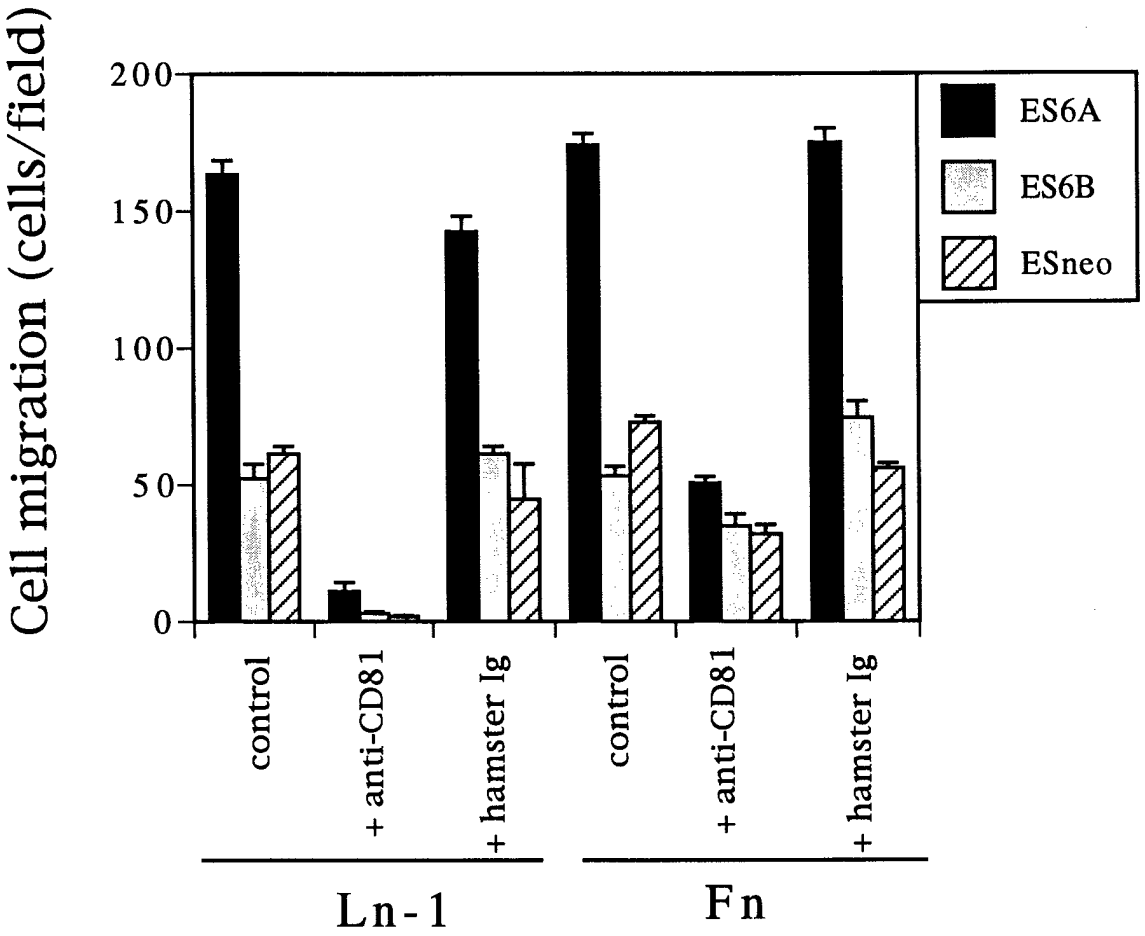
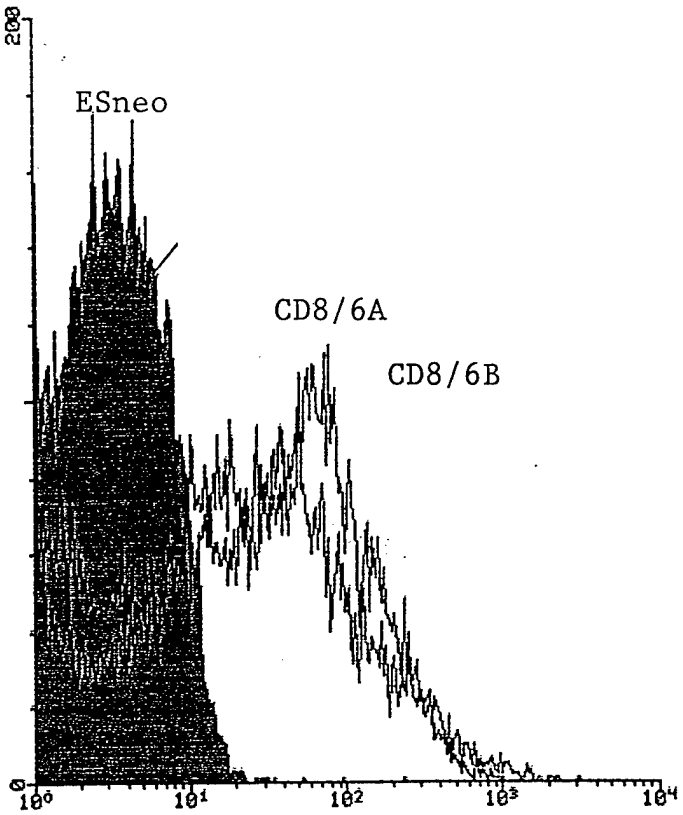
anti CD81

Anti-CD81 does not block adhesion to Ln-1 or Fn

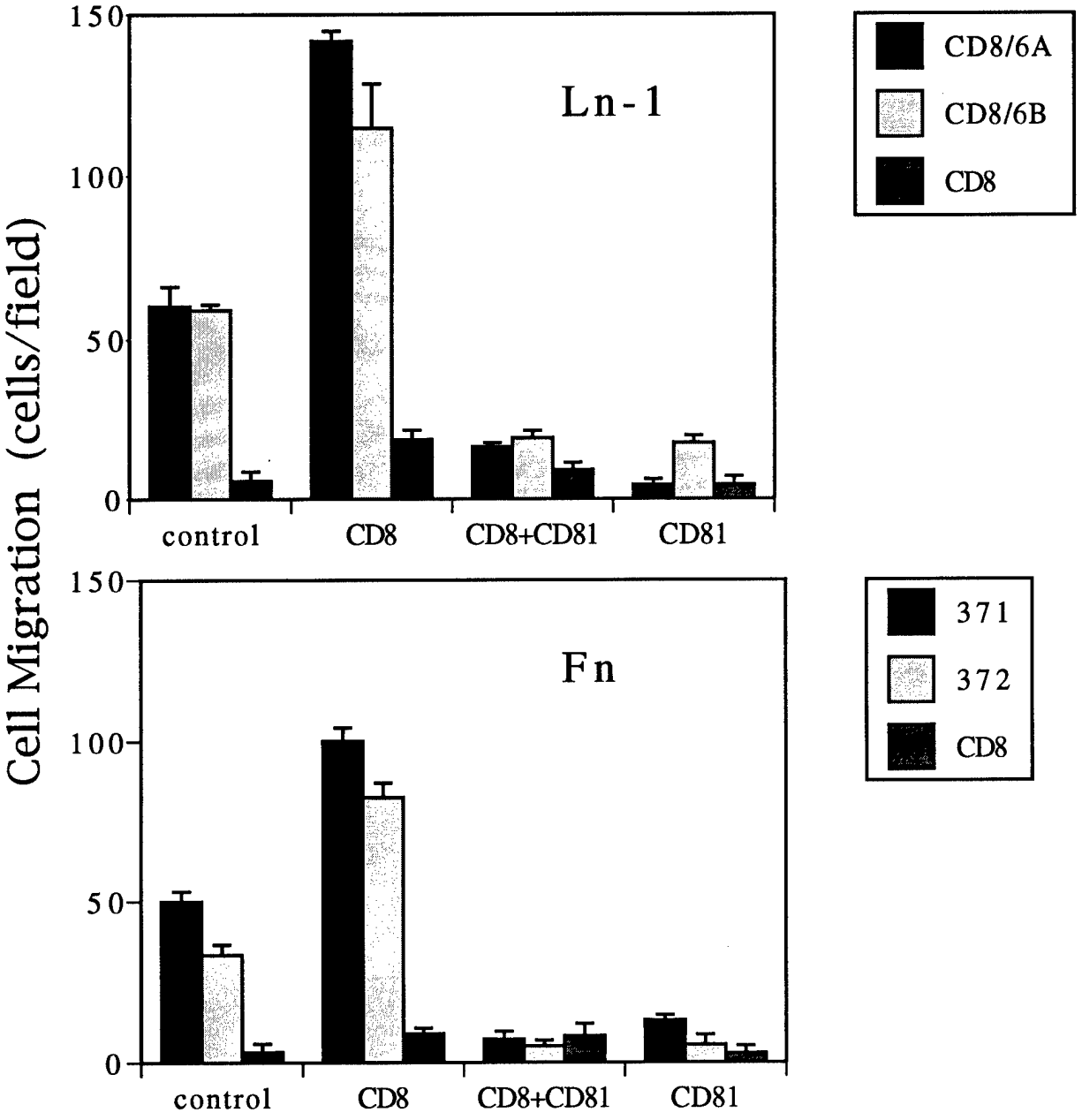


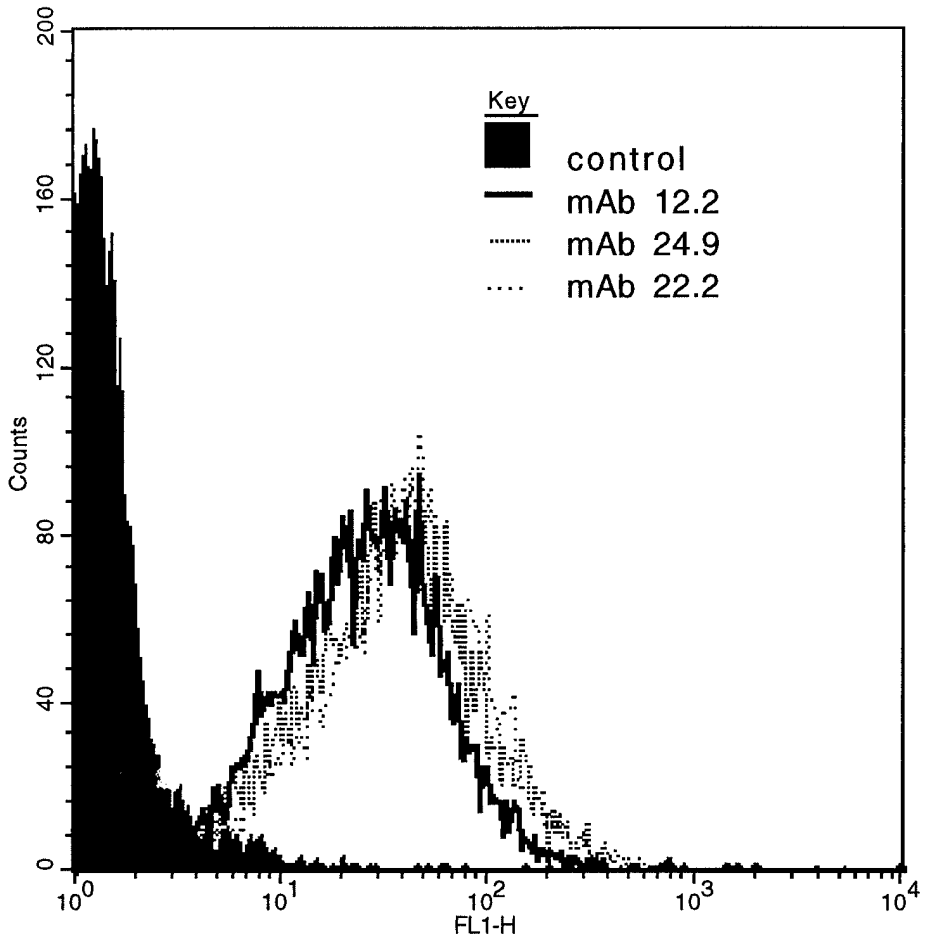
Anti-CD81 antibody blocks migration on Ln-1 and Fn




200
ESneo
CD8/6A
CD8/6B
0
10⁰
10¹
10²
10³
10⁴
Fluorescence

Anti-CD81 blocks migration induced by cross-linking CD8/6A and CD8/6B chimeras

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mAb 24.9
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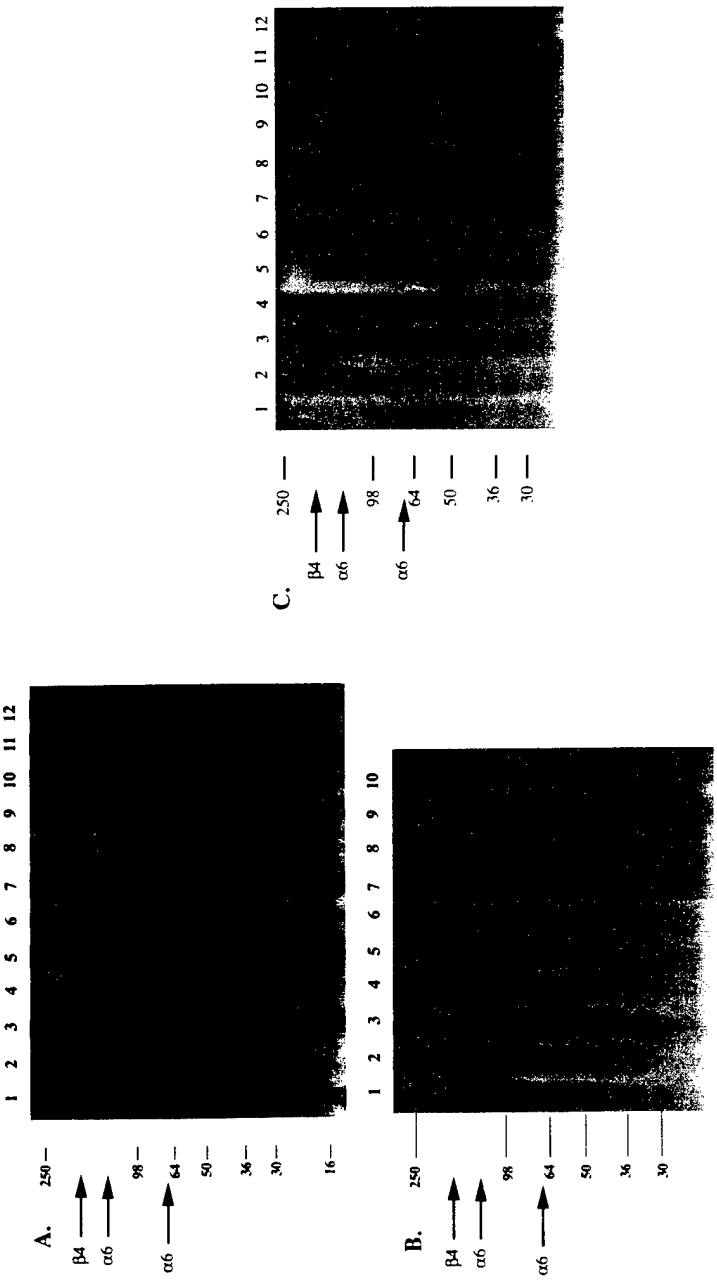
FL1-H


A.
B.
C.
β4
α6
α6
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64
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1 2 3 4 5 6 7 8 9 10 11 12

Publications during funding period:

Domanico, S. Z., Pelletier, A. J., Havran, W. L., and Quaranta, V. (1997) Integrin alpha 6A beta 1 induces CD-81 dependent cell motility without engaging the extracellular matrix migration substrate. Molecular Biology of the Cell. 8:2253-2265.

Abstracts during funding period:

Domanico, S. Z., Pelletier, A. J., and Quaranta, V. (1996) Integrin alpha 6A beta 1 induces cell motility without engaging the extracellular matrix migration substrate. Keystone symposium on cell migration.

Domanico, S. Z., Matter, M., and Quaranta, V. (1995) Upregulation of motility and migration by laminin receptor integrin α6a cytoplasmic domain. Molecular Biology of the Cell. ASCB 1995.

Personnel receiving salary:

Susan Z. Domanico